

Original article

Reduced clearance of respiratory syncytial virus infection in a preterm lamb model

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Received 3 March 2004; accepted 20 August 2004

Available online 02 October 2004

Abstract

Respiratory syncytial virus (RSV) causes significant respiratory disease in children worldwide. For the study of severe RSV disease seen in preterm infants, a suitable animal model is lacking. The novel hypothesis of this study was that preterm lambs are susceptible to bovine RSV (bRSV) infection, an analogous pneumovirus with ruminant host specificity, and that there would be age-dependent differences in select RSV disease parameters. During RSV infection, preterm lambs had elevated temperatures and respiration rates with mild anorexia and cough compared to controls. Gross lesions included multifocal consolidation and atelectasis with foci of hyperinflation. Microscopic lesions included multifocal alveolar septal thickening and bronchiolitis. Immunohistochemistry localized the RSV antigen to all layers of bronchiolar epithelium from a few basal cells to numerous sloughing epithelia. A few mononuclear cells were also immunoreactive. To assess for age-dependent differences in RSV infection, neonatal lambs were infected similarly to the preterm lambs or with a high-titer viral inoculum. Using morphometry at day 7 of infection, preterm lambs had significantly more cellular immunoreactivity for RSV antigen ($P < 0.05$) and syncytial cell formation ($P < 0.05$) than either group of neonatal lambs. This work suggests that perinatal RSV clearance is age-dependent, which may explain the severity of RSV infection in preterm infants. The preterm lamb model is useful for assessing age-dependent mechanisms of severe RSV infection.

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Keywords: Animal models; Antigens; Premature infant; Respiratory syncytial virus

1. Introduction

Respiratory syncytial virus (RSV) infection is a globally important respiratory disease of infants and children. In the United States alone, RSV infection is the most common cause of respiratory disease leading to hospitalization in children, costing an estimated \$300–\$400 million annually [1,2]. RSV is the major cause of bronchiolitis in children, and the incidence of bronchiolitis hospitalization in children under 1 year of age increased 2.4-fold from 1980 to 1996 [3,4]. Infection is ubiquitous and most children have been infected

by 2 years of age [5]. Currently, there is no efficacious treatment for severe RSV infection, and supportive treatment during hospitalization includes bronchodilators, fluid therapy, and oxygen management, with more severe cases receiving corticosteroids or antiviral agents (e.g. ribavirin) [6]. Vaccine development has been slow and cautious following a 1960s vaccine trial in which some vaccinees developed severe disease following natural RSV exposure [7]. Recent developments include a humanized monoclonal antibody (palivizumab) against the fusion (F) protein, which can prevent severe manifestations of RSV disease that require hospitalization, but it does not prevent infection. Because of the high costs of individual treatment, the use of palivizumab is often restricted to high-risk patients. Patients with increased risk for hospitalization and mortality due to severe RSV disease include preterm and young neonatal infants [3,7,8].

Abbreviations: bRSV, bovine respiratory syncytial virus; hRSV, human respiratory syncytial virus; RSV, respiratory syncytial virus.

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Currently, there is no well-recognized model for the study of the severe RSV disease associated with preterm and young neonatal infants. Research of severe RSV infection in infants is limited by technical difficulty, practical and ethical concerns [9]. Study of RSV infection in animal models requires comparable clinical disease, lesion development, immunity, or other features that relate to the specific aims of the experiment [10]. Animal models for study of human RSV (hRSV) pathogenesis and immune response include rats [11], mice [12], ferrets [13], guinea pigs [14], hamsters [15], lambs [16] and nonhuman primates [17]. Infection in the chimpanzee is viewed by some to be the most analogous to human disease; however, animal availability is limited, few are naive and they require specialized facilities and management, making this model impractical [18]. Mice are often used in models for human disease (including RSV) because of the ease of handling and availability of strains with specific knockout genes [12]. However, hRSV infection in rodents produces only mild bronchitis without overt respiratory disease, making distinctions about severe infection difficult.

Late-term fetal and preterm lambs have proven valuable as pulmonary models for the study of surfactant expression and regulation [19], ventilation-induced injury [20], congenital diaphragmatic hernia [21], chorioamnionitis [22], sleep apnea [16] and persistent pulmonary hypertension of the newborn [23]. A preterm lamb model of hRSV was previously evaluated for RSV-induced sleep apnea, and infection yielded only mild clinical disease [16]. An analogous virus model is bovine RSV (bRSV), a pneumovirus that is genomically, antigenically and functionally related to hRSV and is host-adapted for ruminant infection causing significant pulmonary lesions and clinical disease [18,24,25]. The preterm lamb model of bRSV infection has not been assessed for use as a model of RSV disease and lesions. Previous work in the sheep suggested, bRSV infection may cause more severe lesions in neonatal versus 6-month-old lambs [25,26]. Furthermore, study of hRSV infection in senile cotton rats suggested age-dependent mechanisms for RSV clearance [11]. Since developmental age is a determinant in severity of RSV disease and lesions, bRSV infection in the preterm lambs could be a novel model for studying the severity of RSV disease in preterm infants. The hypothesis of this study was that preterm lambs are susceptible to bRSV infection and that bRSV activity would be age-dependent in the perinatal lamb.

2. Materials and methods

2.1. Animals

Healthy, date-mated commercial ewes were acquired from Iowa State University's Laboratory Animal Services. A gestational age of 138 days (term, 147 days) was chosen to provide a preterm lamb, which would not require mechanical ventilation, a known instigator of pulmonary injury [20]. Following removal via surgical uterotomy, lambs were given

tactile stimulation with manual chest compressions (if necessary) until respiration was self-regulated, then dried and placed in a thermo-regulated pen. Within the first hour, fresh colostrum (~200 ml) was given via a stomach tube, and ceftiofur (2.2 mg/kg per day, intramuscular) was given to prevent potential bacterial complications [27]. Lambs were hand-fed commercial milk replacer four times daily and milk consumption recorded. In addition, body temperatures and respiration rates were recorded at the same time each day following the morning feeding. Lambs were inoculated at 1 day of age as follows: the cervical midline was sanitized with 70% ethanol and the trachea isolated for intratracheal injection (20 cm³) of viral inoculum ($n=6$) or sterile media ($n=6$).

In addition, 11 neonatal lambs (2–4 days of age) were acquired from Iowa State University's Laboratory Animal Resources and inoculated with bRSV similarly to the preterm lambs, 1 day following arrival. Five of the lambs were inoculated with the same virus inoculum as preterm lambs, three lambs received high-titered viral inoculum and the remaining three received sterile media as a control.

2.2. Virus

bRSV strain 375 was grown in flasks containing adherent bovine turbinate cells (5% CO₂ and 37 °C). When 90% of virus-induced cytopathic effect was visible (usually within 7 days), the flasks were frozen (–80 °C) and then thawed the next day. All flask media were pooled, thoroughly mixed, sterile filtered and aliquoted. The aliquots were stored at –80 °C. One aliquot was used to determine the tissue culture infective dose (TCID₅₀ per ml) by standard plaque assay, and the virus titer ranged from 10^{3–4} TCID₅₀ per ml. For the second group of neonatal lambs, further passages were made to produce a high-dose inoculum with a titer 10⁷ TCID₅₀ per ml.

2.3. Tissue

On day 7 of infection, lambs (preterm and neonatal) were euthanized with an intravenous injection of sodium pentobarbital. From our preliminary work with bRSV in neonatal lambs, day 7 was chosen because this is approximately when the most severe lesions are seen and when clearance of RSV antigen is under way [28]. The thorax was opened and the lungs examined for gross lesions. The lungs were then removed from the thorax for subsequent tissue collection. The lung was consistently sampled from the left and right: cranial, middle and caudal lobes. Tissues were placed in 10% buffered formalin and processed routinely for hematoxylin and eosin staining or immunohistochemistry.

2.4. Immunohistochemistry

Sections of lung on silanated glass slides were stained with antibody to RSV antigen using a streptavidin–biotin

peroxidase method developed in our laboratory. Briefly, slides of lung tissue were heated to 58 °C for 20 min and deparaffinized through a series of ethanol and xylene baths. Antigen retrieval was performed by applying “Pronase E” (0.1 g Protease XIV (Sigma, St. Louis, MO) and 0.1 g CaCl₂ per 100 ml TBS pH 7.6) on heated slides for 10 min followed by Tris (pH 7.6) (5×) and PBS (Biogenex, San Ramon, CA) washes (2×). Normal swine serum (20% for 20 min) was applied as a nonspecific blocker followed by primary antibody (mouse monoclonal anti-bRSV-4 (courtesy of Dr. Ken Platt, Iowa State University), 1:100, with normal swine serum (5%) and normal goat serum (5%)) for 72 h at 4 °C. Slides were warmed to room temperature followed by multiple PBS washes. Endogenous peroxidases were blocked by a 40-min application of hydrogen peroxide (3%) in PBS and then repeated PBS washes. Secondary antibody (1:300 biotinylated goat anti-mouse (Kirkegaard & Perry Laboratories, Gaithersburg, MD) with normal swine serum (15%)) was applied for 45 min followed by multiple PBS washings. Next, the slides were treated with streptavidin-conjugated horseradish peroxidase (Biogenex, 45 min), multiple PBS washes, and the chromogen Nova Red (Vector, Burlingame, CA, 5 min). The slides were washed in PBS, counterstained with Harris’ hematoxylin, dehydrated through a series of ethanol and xylene baths and were coverslipped.

2.5. Morphometry

The purpose of the morphometry was to quantify the number of cells staining for bRSV antigen in lesions. Sections of lung from the right and left cranial lobes were used for the study. Since bRSV lesions and immunoreactivity were principally in and around small bronchioles, a pathologist (blinded from the study) randomly selected from low-power bronchioles within lesions. At higher magnification, a grid [29] was centered over the bronchiole and adjacent tissue with the total number of positive cells and morphological cell-type was recorded (area = 440 × 440 µm (10^{5.3} µm²)). At this same time, counts for syncytial cells and mitosis were performed. A total of 10 bronchiolar lesions were counted from the left and right sides and averaged.

2.6. Statistics

For clinical data, the group means on each day were analyzed for significance using pair-wise *t*-tests. In the morphometry experiments, the resulting data was analyzed using the Wilcoxon–Mann–Whitney test. For all data, significance was placed at *P* < 0.05.

3. Results

Clinical scores for the lambs included body temperature, respiration rate and milk consumption (Table 1). The infected preterm lambs had slightly higher temperatures than those of

Table 1

Average body temperature, respiration rate and daily milk consumption by control and RSV-infected preterm lambs

Day of infection	Group	Temperature (°C ± S.E.M.)	Respiratory rate (respirations per min ± S.E.M.)	Daily milk consumption (ml ± S.E.M.)
Day 1	Control	39.44 ± 0.17	61 ± 6.3	–
	RSV	39.69 ± 0.11	57 ± 1.3	–
Day 2	Control	39.74 ± 0.06	64 ± 3.3	563.3 ± 102.0
	RSV	39.98 ± 0.05 *	60 ± 2.2	498.3 ± 47.8
Day 3	Control	39.57 ± 0.13	60 ± 3.8	607.5 ± 84.6
	RSV	39.95 ± 0.05 *	65 ± 2.9	583.5 ± 78.3
Day 4	Control	39.69 ± 0.06	59 ± 1.8	815.8 ± 107.7
	RSV	39.78 ± 0.07	64 ± 4.8	657.5 ± 80.5
Day 5	Control	39.59 ± 0.11	50 ± 3.0	841.7 ± 100.3
	RSV	39.63 ± 0.16	68 ± 5.0 *	676.7 ± 63.3
Day 6	Control	39.7 ± 0.1	52 ± 4.0	892.5 ± 91.1
	RSV	39.78 ± 0.05	64 ± 3.3 *	764.2 ± 69.7
Day 7	Control	39.69 ± 0.04	49 ± 1.8	903.3 ± 106.2
	RSV	39.87 ± 0.15	55 ± 1.8	785.8 ± 103.6

* RSV group compared to control group (*P* < 0.05).

control lambs throughout the infection, with significantly higher temperatures on days 2 and 3 of infection (*P* < 0.05 versus control lambs). In the infected group, temperatures peaked on days 2–3 of infection (39.98 ± 0.05 and 39.95 ± 0.05 °C, respectively, Table 1) with a decrease until day 5 with another slight increase starting on days 6–7 of infection. From day 3 through the course of the infection, respiratory rates for the infected group were slightly higher than controls, with significant elevation on days 5 and 6 of infection (*P* < 0.05 versus control lambs). Milk consumption was slightly reduced in the bRSV group compared to the control group through the course of the infection. Infected lambs had a mild cough most noticeable upon exertion.

In regards to preterm lambs and susceptibility to bRSV, all of the infected lambs developed lung lesions, while the control lungs were normal. Grossly, the lungs had multiple plum-red foci (2–10 mm) of consolidation that was slightly depressed from the adjacent pleural surface. On cut-surface, the lesions were slightly firm with well-defined borders. The lesions were most evident on the ventral half of the lungs with accentuation in the cranial and hilar regions. Adjacent to the consolidated lesions were pale-pink hyperinflated areas of lung.

Microscopically, there were multifocal areas in which alveolar septa were thickened by macrophages, lymphocytes, plasma cells with small numbers of neutrophils and moderate vascular congestion (Figs. 1–3). Intralesional bronchioles had luminal neutrophils, sloughed epithelial cells and lesser amounts of macrophages with eosinophilic cellular and karyorrhectic debris (Fig. 4). Bronchiolar epithelium was often thickened by hyperplasia of epithelial cells, many of which had mitotic figures. There were also multiple moderate infiltrates of neutrophils and areas of necrosis. In addition, bronchioles had a small number of individual apoptotic cells usually within cytoplasmic vacuoles of adjacent epithelia or mononuclear cells. A small number of the epithelial cells

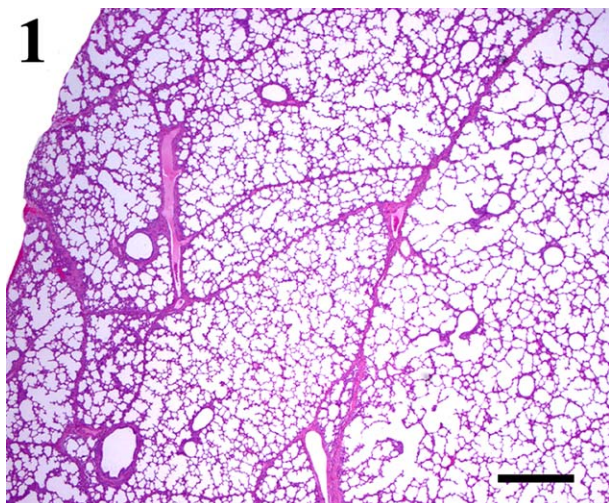


Fig. 1. Control lungs from lambs given sterile media lacked lesions; bar = 500 μ m.

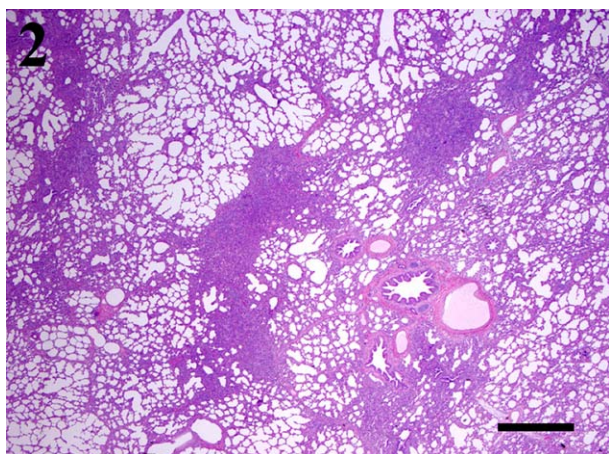


Fig. 2. bRSV-infected lungs had multiple foci of bronchointerstitial consolidation; bar = 500 μ m.

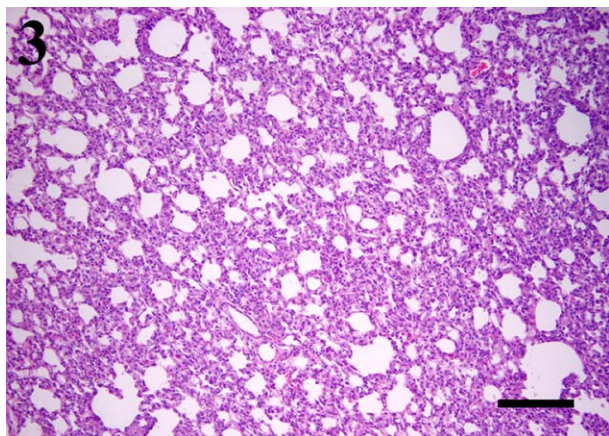


Fig. 3. Within lesions, the alveolar septa were thickened by predominantly mononuclear infiltrates; bar = 100 μ m.

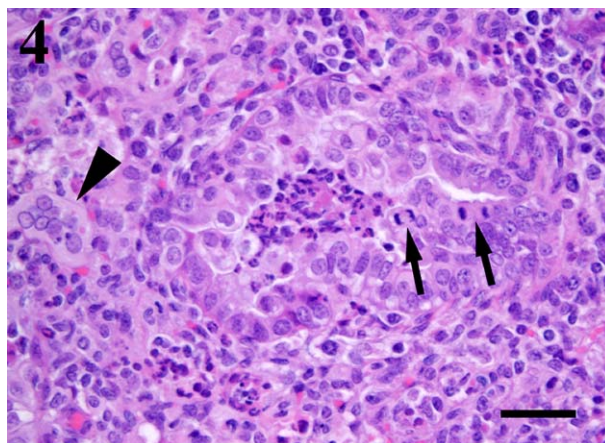


Fig. 4. Infected bronchiolar epithelium was thickened by hyperplasia with multiple mitotic figures (arrows) and neutrophilic necrotic cellular debris in the lumen. Note the syncytial cell formation (arrowhead); bar = 25 μ m.

were multinucleated (as syncytial cells) and/or contained small (3–5 μ m) intracytoplasmic eosinophilic structures consistent with viral inclusion bodies.

Immunohistochemistry was used to confirm the presence of bRSV antigen and localize the sites of cellular replication. Sections of control lung were consistently negative. In infected lung, all levels of bronchiolar epithelium were immunoreactive for bRSV antigen, including a few basal cells to several sloughing epithelial cells (Fig. 5). Within lesions, a few mononuclear cells were immunoreactive, and most had vacuolated to foamy cytoplasm that were morphologically consistent with macrophages. The granular cytoplasmic staining of immunoreactive epithelial and mononuclear cells was characterized by small globular structures (~0.5–8 μ m) within the cytoplasm and was consistent with viral inclusions. A smaller number of neutrophils contained antigen. These were often degenerate, found only in the lumen of connecting airways and lacked distinct globular structures (inclusions) seen in other infected cells.

Cellular immunoreactivity, syncytial cell formation and mitotic index was used to assess RSV activity and host repair in the preterm versus neonatal lamb. The preterm lambs had

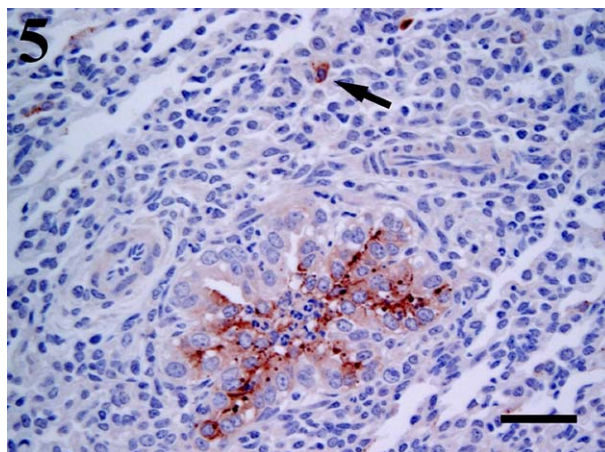


Fig. 5. Staining for bRSV antigen was primarily within bronchiolar epithelium and a few mononuclear cells (arrow); bar = 25 μ m.

more cellular reactivity for bRSV antigen than did neonatal lambs with similar inocula ($P < 0.05$) or high-titer inocula ($P < 0.05$) (Fig. 6A). Two out of the five lambs in neonatal group 1 (similar virus titer) and one out of three lambs in neonatal group 2 (high virus titer) lacked evidence of immunoreactivity, while all of the preterm infected lambs had ample bRSV antigen. The syncytial cell formation, indicative of RSV activity, was significantly higher in the preterm group compared to either of the neonatal bRSV groups ($P < 0.05$) (Fig. 6B). The mitotic rate, an indicator of reparative hyperplasia, was similar between the different groups (Fig. 6C).

4. Discussion

The hypothesis of this study was that preterm lambs would develop bRSV disease and that bRSV antigen distribution at the time of clearance would be more widespread in preterm versus full-term neonatal lambs. The RSV-infected preterm lambs showed clinical evidence of infection including elevated temperature and increased respiration rate with mild loss of appetite (milk consumption) and cough. The pyrexia and tachypnea are consistent with previous work in older neonatal lambs with hRSV or bRSV infection [16,25]. Infants with severe hRSV infection typically exhibit a low-grade to moderate fever, croup-like cough and tachypnea with anorexia, lethargy or irritability [3]. The clinical signs in the preterm lambs paralleled many features of severe hRSV infection in infants but notably lacked the severe croup-like cough. This lack of severe cough is similar to previous work in lambs with bRSV [30], but may also be, in part, a consequence of the mid-tracheal inoculation, which would have minimized exposure of the upper tracheal and laryngeal epithelium to bRSV for development of laryngotracheitis.

All infected lambs had gross lesions, while the controls lacked lesions. The cranioventral to hilar distribution of the lesions was likely a partial result of the aspiration-like allocation of viral media following inoculation and is similar to other work [28]. Gross lung lesions were composed of multifocal atelectasis and consolidation with hyperinflation. Infants with severe hRSV infection can have radiographic evidence of interstitial lung patterns with foci of atelectasis, peribronchiolar thickening and hyperinflation, which corresponds to lesions in this study [31].

Histologically, a bronchointerstitial pattern was seen in multiple foci with primarily a mononuclear (macrophages and lymphocytes) infiltrate and congestion in alveolar septa. Severe RSV infection in infants is associated with elevated chemokine levels including RANTES (CC chemokine ligand 5 (CCL5)) and macrophage inflammatory protein-1 α (CCL3), which are chemotactic for monocytes and other leukocytes [31–33]. RSV-induced chemokine expression likely provided the means for the mononuclear leukocyte infiltrate.

The intralesional bronchioles were characterized by epithelial sloughing, necrosis, and hyperplasia with infiltrates of

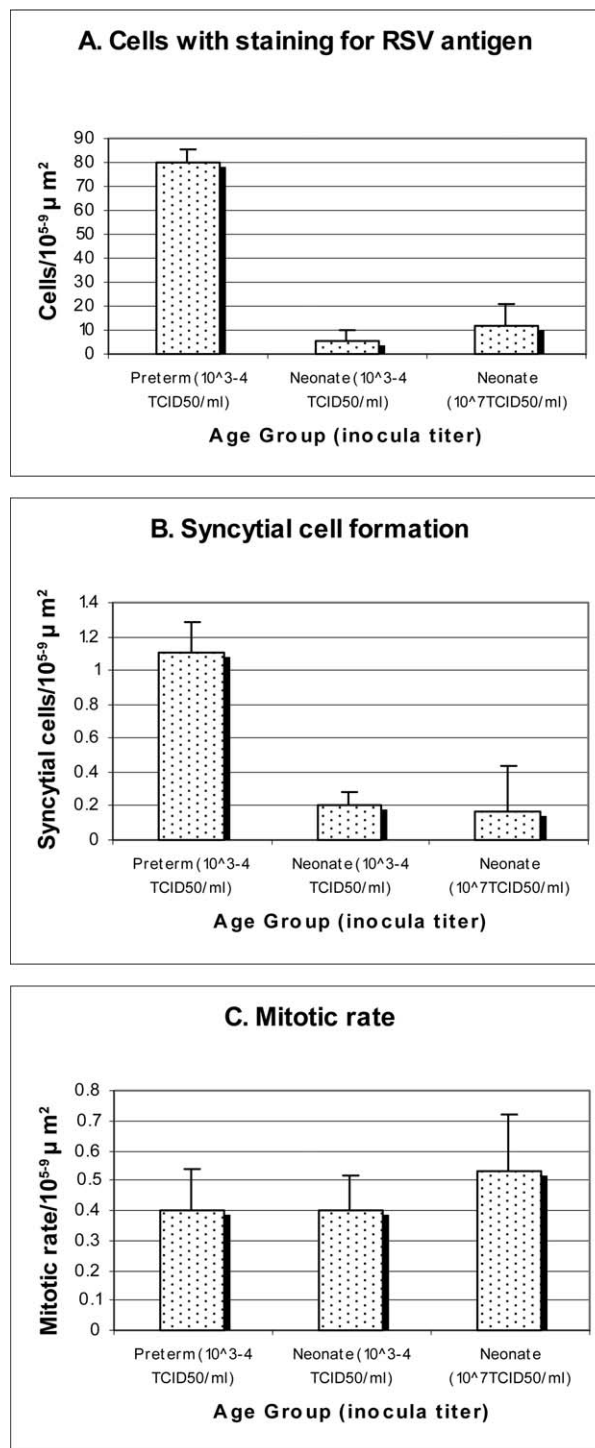


Fig. 6. Morphometry of cellular staining for bRSV antigen, syncytial cell formation and mitotic rate in bRSV-inoculated preterm ($20 \text{ ml} \times 10^{3-4}$ TCID₅₀ per ml) and neonatal ($20 \text{ ml} \times 10^{3-4}$ TCID₅₀ per ml or 10^7 TCID₅₀ per ml) lambs. **A:** The cellular staining for bRSV antigen was greater in the preterm versus either neonate group ($P < 0.05$). All preterm lambs had strong staining, while the neonate group had reduced individual staining, including two of five lambs in neonate- 10^{3-4} TCID₅₀ per ml and one of three lambs in neonate- 10^7 TCID₅₀ per ml, which were negative for staining. **B:** Syncytial cell formation, indicative of bRSV activity, was greater in the preterm lambs compared to either of the neonate groups ($P < 0.05$). **C:** The mitotic rate, a marker for the degree of reparative hyperplasia, was similar between the preterm and neonatal groups.

neutrophils. Leukocyte populations of the connecting airway in severe RSV infection are typically composed of neutrophils, and they participate in the viral immunopathology [34]. Epithelial sloughing and necrosis is, in part, a direct result of neutrophil adhesion and exocytosis [35]. RSV infection of epithelial cells and monocytes leads to a synergistic up-regulation in expression of interleukin-8 (IL-8), a CXC chemokine that enhances neutrophilic chemotaxis, and enhanced IL-8 expression is correlated with severe RSV infection [36,37]. An additional source for epithelial cellular debris is apoptosis, a common mechanism for clearance of RSV-infected epithelia [27].

Adjacent bronchiolar epithelium was hyperplastic, which is indicative of epithelial repair [38]. The mitotic rate, an indicator of the degree of hyperplasia, was similar between the preterm and neonatal lambs. This present study suggests that reduced repair capacity is not likely a factor in severe RSV disease.

Staining for bRSV antigen was present only in infected lambs and was localized to the bronchiolar epithelium with some mononuclear cells. Recently, putative receptor(s) for RSV was described to have L-selectin-like properties [39]. L-Selectin is a member of the adhesion-molecule selectin family and is important in leukocyte tethering for endothelial transmigration during normal trafficking or at sites of inflammation [40]. One of the proposed RSV receptors was further characterized as annexin II [39]. Annexin II belongs to the annexin family of calcium- and phospholipid-binding proteins. In the lung, annexin II is selectively expressed in basal cells, but not columnar cells of the respiratory epithelium [41]. Infection of basal cells would be advantageous for RSV biologically in allowing adequate time during normal cell turnover for viral replication. While a few basal cells contained antigen, most staining was in the sloughing epithelial cells, which may correspond to the chronological turnover of previously infected basal cells at inoculation.

The immunoreactivity in mononuclear cells and neutrophils could be due to direct virus infection or phagocytosis of infected cellular debris. Direct infection is possible via putative receptors such as L-selectin-like moieties found on most leukocytes or annexin II, which has been localized to monocytes [40,42]. Ultrastructurally, macrophages of calves infected with bRSV show viral inclusions in the cytoplasm consistent with infection [43]. Most of the mononuclear cell staining in this current study was morphologically consistent with macrophages, and given the intracytoplasmic viral inclusions, direct RSV infection of macrophages was most likely. Other mononuclear cells such as dendritic cells and lymphocytes cannot be excluded, as *in vitro* studies have shown capacity for RSV uptake [44,45]. As for neutrophil antigen accumulation, phagocytosis of sloughed infected cellular debris in airways probably allowed for much of the neutrophil staining, since there was a lack of distinct cytoplasmic inclusions, and the quick demise of the degenerate neutrophil following exocytosis into the airway lumen would prevent opportunity for adequate viral replication.

Preterm lambs had significantly more immunoreactive cells and syncytial cell formation than did neonatal lambs with similar (10^{3-4} TCID₅₀ per ml) or even higher (10^7 TCID₅₀ per ml) virus inoculum. The timing of RSV clearance, as assessed by virus isolation, closely parallels staining for bRSV antigen by immunohistochemistry [28]. Syncytial cell formation is, in part, a by-product of the RSV fusion (F) protein and an indicator of RSV activity [46]. The increased bRSV immunoreactivity and syncytial cell formation in the preterm lambs are indicative of a reduced capacity to clear the virus compared to the neonatal lambs. Similar age-dependent differences in viral clearance were seen while studying severe hRSV disease, as senile versus young adult cotton rats were less efficient at viral clearance [11]. Reduced RSV clearance in the preterm infant would prolong and perhaps exacerbate inflammation, leading to severe clinical disease.

Viral clearance in bRSV-infected calves is primarily mediated through apoptosis of infected cells with phagocytic removal of the apoptotic bodies by adjacent epithelial cells or monocytic cells [27]. In cultured respiratory epithelia, RSV infection directly results in expression of many pro- and anti-apoptotic factors. The intracellular balance of these factors may be pushed towards apoptosis through external mononuclear-induced cytotoxicity by binding of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) to death receptor-4/5 (DR4/5) [47]. TRAIL is quickly expressed on monocytes following interferon- α or - γ activation (common with RSV infection), and subsequent binding of DR4/5-expressing cells causes induction of apoptosis [48]. Interestingly, lamb monocytes infected with bRSV have decreased phagocytosis and antigen-presentation ability [49]. In addition, preterm lamb monocytes have delayed hydrogen peroxide production and reduced phagocytosis of apoptotic cells compared to monocytes from neonatal lambs [50]. Decreased monocytic function in the RSV-infected preterm infant may contribute to lack of RSV clearance and thus allow for severe RSV disease.

In this study, we highlight the novel use of bRSV infection in the preterm lamb, which exhibits clinical signs and comparable lesions to severe hRSV disease in the preterm infant. Furthermore, preterm lambs have reduced capacity for RSV antigen clearance compared to neonatal lambs. The preterm lamb model is useful to study age-dependent differences seen in severe RSV infection of preterm infants.

Acknowledgments

The authors would like to thank Rachel Derscheid, Erin Costello and Dr. Vanessa Prest for technical assistance. This work was funded by a USDA/CSREES/NRI/CGP grant, award number 2003-35204-13492.

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